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Integration of the Bacteriophage φ3T-Coded Thymidylate Synthetase Gene into the *Bacillus subtilis* Chromosome

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Transformation of *Bacillus subtilis* 168 Thy⁻ auxotrophs with φ3T deoxyribonucleic acid (DNA) to thymine independence was found to involve site-specific recombination of φ3T DNA sequences with their homologous counterparts in the bacterial chromosome. During the transformation, the phage φ3T-encoded thymidylate synthetase gene, *thyP3*, was shown to integrate at two genetically distinct sites in the *B. subtilis* 168 chromosome. The first site was identified to be in the bacterial thymidylate synthetase gene, *thyA*. The second site was in a prophage (SPB) known to be carried in the host genome. The frequency of the integration of the *thyP3* gene at each of the two loci and some of the parameters affecting this frequency were studied. The common origin of the *thyP3* and *thyA* genes and their molecular evolution are also reported.

Transformation of bacteria of a given species by DNA from other organisms has been termed heterologous or interspecies transformation. It occurs in *Bacillus subtilis* with very low frequency, even if the transforming DNA is derived from a closely related species (5, 14). A known exception is transformation of *B. subtilis* thymine auxotrophs to prototrophy by DNA extracted from its temperate bacteriophage, φ3T (11). φ3T, which carries a structural gene for thymidylate synthetase, designated *thyP3* (3), can be propagated equally well on Thy⁻ and Thy⁺ *B. subtilis* strains. Its DNA transforms *B. subtilis* almost as efficiently as homologous bacterial DNA (11). The transformation of Thy⁻ bacteria to the Thy⁺ phenotype does not require the integration of the entire bacteriophage genome into the chromosome (15), and thus it bypasses simple lysogenization. Young et al. (15) reported that in one *B. subtilis* transformant, *thyP3* integrated in the vicinity of the φ3T attachment site, which is in a chromosomal region distinct from the location of the two genes encoding thymidylate synthetases in *B. subtilis*, *thyA* and *thyB*.

We have recently shown that the *B. subtilis* chromosome contains several regions homologous to φ3T DNA which might be potentially involved in site-specific recombination with the *thyP3* gene (10). To explore this possibility and understand the mechanism underlying transformation by φ3T DNA, several *B. subtilis* auxotrophs were transformed to thymine independence by the *thyP3* gene and were analyzed. Two integration sites were identified. In addition, the three thymidylate synthetase genes, *thyA*, *thyB*, and *thyP3*, were examined with the hope of understanding any possible evolutionary relationships among them. Thymidylate synthetase genes in *B. subtilis* are of particular interest since this bacterium is the only known procaryotic organism that contains two thymidylate synthetases with very similar catalytic activities. Thymidylate synthetase B appears to play a minor role in thymidine nucleotide biosynthesis under physiological conditions in *B. subtilis* (6). It is unknown why both *thy* genes have been retained in this species during evolution. Mutations in both *thyA* and *thyB* are necessary to create an absolute thymine requirement, and reversion in either one of them is sufficient to suppress thymine auxotrophy (13). The two prototrophic mutants can, however, be distinguished phenotypically. A *thyA thyB*⁺ mutant is partially resistant to trimethoprim and aminopterin in the presence of thymine. In addition, a *thyA thyB*⁺ mutant is capable of incorporating exogenous thymine into DNA and requires thymine or thymidine for growth at 46°C. In contrast, a *thyA*⁺ *thyB* mutant is phenotypically like the wild type: sensitive to antifolates, not affected by high temperature, and unable to utilize exogenous thymine (6).

This paper presents results suggesting that the phage *thyP3* and the bacterial *thyA* loci derive from a common ancestral gene.

MATERIALS AND METHODS

Bacterial strains. All of the *B. subtilis* strains used in this work are derivatives of Spizizen's *B.*

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subtilis 168 (9). SB165 (*trpC2*), SB591 (*thyA thyB*), and SB1141 (*thyA trp ilvD6*) are from the Stanford University collection; SB1200 (*thyB ilvA8 citB1 gapA2*) and SB1207 (*leu met thr* SPβ^-) were obtained from S. Zahler, and SB1123 (ϕ3T lysogen of SB168) was obtained from D. Dean. SB1219 (*thyA thyB leu* SPβ^-) was constructed by transformation of SB1207 with DNA extracted from SB591, followed by trimethoprim selection (12). SB1223 (*thyB leu* SPβ^-) is a thymine-independent, spontaneous revertant of SB1219. Table 1 lists other derivatives of SB591.

pFT plasmids were propagated in *Escherichia coli* strain W5443 (*hsdR hsdM leu thi thy rspL trp tonB*) or W5545 (*hsdR hsdM*$^+$ *thr leu thi supE44 rspL lac tonA pro*). Their molecular structure is summarized in reference 2, in the accompanying paper (10), and in Table 1.

Enzymes and reagents. Restriction enzymes *Hin*dIII, *Bam*HI, *Bgl*II, and *Hae*I were purchased from Biolabs Inc. *Eco*RI was purchased from Miles Laboratories, Inc. The digestions were done according to the specifications recommended by the vendors of the enzymes. Trimethoprim was purchased from Calbiochem. Other materials and methods are described in the accompanying paper (10).

RESULTS

Evidence for two integration sites. A series of strains was constructed by transformation of *B. subtilis* thymine auxotrophs with ϕ3T DNA, with *Bam*HI-cleaved ϕ3T DNA, or with chimeric plasmids pFT23, -24, -25, -33, -34, -401, -451, and -603. The molecular structure and properties of most of these recombinant plasmids have been described elsewhere (10; Table 1). All chimeras carry overlapping inserts of ϕ3T DNA in different *E. coli* vectors. The size of the inserts varies from 2.1 to 6.3 megadaltons (Mdal). The region of overlap includes the *thyP3* gene.

In the transformants analyzed, the *thyP3* gene integrated at two different sites in the *B. subtilis* chromosome (Fig. 1). DNA from SB168-derived strains SB1200 (*thyA*$^+$ *thyB*) and SB591 (*thyA thyB*) as well as from several *thyP3*$^+$ transformants of SB591 was digested with the *Hin*dIII enzyme, electrophoresed in 0.7% agarose gels, and transferred to nitrocellulose filters (8). RNA complementary to pFT *thyP3* (described in reference 10) hybridized to a 1.6-Mdal *Hin*dIII DNA band of SB1200 (*thyA*$^+$ *thyB*) in channel A and to a 1.5-Mdal *Hin*dIII band of SB591 in channel B. This is consistent with the assumption that the Thy$^-$ phenotype of SB591 is the result of a small deletion (~0.1 Mdal) in the region of the chromosome containing the *thyA* locus. The existence of a *thyA* deletion in SB591 was independently confirmed by the analysis of spontaneous Thy$^+$ revertants. SB591 reverted with a frequency 10^{-8}. Each of the 30 revertants analyzed behaved phenotypically like *thyA thyB*$^+$ mutants; that is, they were partially resistant to trimethoprim in the presence of thymine. This is consistent with the assumption that SB591 can revert at *thyB* but not at the deleted *thyA* locus. Only a few of the revertants were temperature sensitive like the wild-type *thyB* (2 of 30). Some were partially temperature resistant (10 of 30). However, the majority were heat resistant. These clones were not studied further. Channels C, E, F, G, and H of Fig. 1 contain *Hin*dIII-cleaved DNA of strain SB591 transformed to thymine independence with ϕ3T DNA (SB1150) and chimeric plasmids: pFT34

TABLE 1. *Derivatives of SB591 (thyA thyB)*[a]

Strain	Genotype	DNA used in transformation of SB591	Comments about transforming *thyP3*$^+$ DNA
SB1149	*thyA thyB thyP3*$^+$		
SB1150	*thyA thyB thyP3*$^+$	ϕ3T	79.1-Mdal linear phage DNA
SB1151	*thyA thyB thyP3*$^+$	pFT23	5.4-Mdal ϕ3T DNA insert in pSC101 (7)
SB1152	*thyA thyB thyP3*$^+$	pFT24	4.5-Mdal ϕ3T DNA insert in pSC101 (7)
SB1155	*thyA thyB thyP3*$^+$	*Bam*HI ϕ3T	36.6-Mdal linear phage DNA
SB1162	*thyA thyB thyP3*$^+$	pFT25	6.3-Mdal ϕ3T DNA insert in pSC101 (7)
SB1163	*thyA thyB thyP3*$^+$	pFT33	7.2-Mdal ϕ3T DNA insert in pSC101 (7)
SB1164	*thyA thyB thyP3*$^+$	pFT34	4.5-Mdal ϕ3T DNA insert in pSC101 (7)
SB1165	*thyA thyB thyP3*$^+$	pFT501	2.1-Mdal ϕ3T DNA insert in pMB9 (7)
SB1166	*thyA thyB thyP3*$^+$	pFT502	2.1-Mdal ϕ3T DNA insert in pMB9 (7)
SB1167	*thyA thyB thyP3*$^+$	pFT401	4.5-Mdal ϕ3T DNA insert in RSF2124 (7)
SB1168	*thyA thyB thyP3*$^+$	pFT402	4.5-Mdal ϕ3T DNA insert in RSF2124 (7)
SB1169	*thyA thyB thyP3*$^+$	pFT603	4.5-Mdal ϕ3T DNA insert in pML2 (7)
SB1170	*thyA thyB thyP3*$^+$	pFT451	2.1-Mdal ϕ3T DNA insert in pSC101 (7)
SB1203	*thyA thyB thyP3*		
SB1204	*thyA thyB thyP3*		

[a] Most of the strains listed above (SB1150 to SB1170) are Thy$^+$ transformants of SB591 obtained with ϕ3T DNA or DNA from ϕ3T-*E. coli* chimeric plasmids. SB1149 is a ϕ3T lysogen of SB591; SB1203 and SB1204 were constructed by lysogenization of SB591 with two different Thy$^-$ derivatives of ϕ3T.

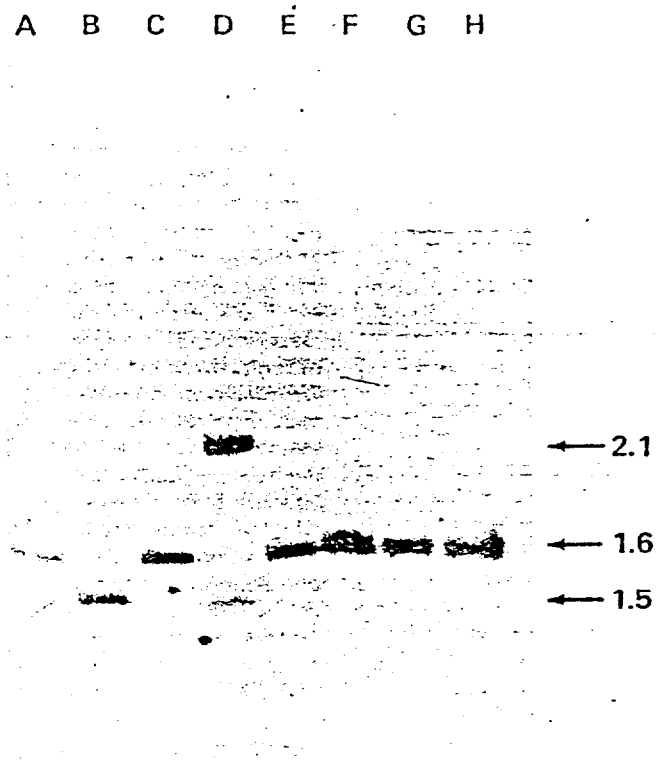

FIG. 1. *Integration of the thyP3 gene into two distinct sites of the B. subtilis chromosome. DNA from (A) SB1200, (B) SB591, (C) SB1150, (D) SB1155, (E) SB1164, (F) SB1167, (G) SB1169, and (H) SB1170 was cleaved with HindIII enzyme, fractionated in 0.7% agarose gels, and transferred to nitrocellulose filters.* ^{32}*P-labeled RNA complementary to pFT thyP3 was used as a probe in this hybridization experiment. Sizes of stained bands are expressed in megadaltons.*

(SB1164), pFT401 (SB1167), pFT603 (SB1169), and pFT451 (SB1170). All of these transformants hybridized complementary RNA (cRNA) pFT *thyP3* to a 1.6-Mdal band which was identical in molecular weight to the band observed in the *thyA*$^+$ strain. A similar result was observed in strains transformed by pFT23 (SB1151), pFT24 (SB1152), pFT25 (SB1162), and pFT33 (SB1163) (data not shown). Apparently, the transformation reintroduced the amount of DNA that was missing in the SB591 deletion, thus restoring the hybridization pattern observed with the wild-type gene.

Further support for the integration of the *thyP3* gene into the *thyA* locus was provided by the experiment described in Fig. 2. The DNA restriction fragments containing the *thyA* gene, *thyB* gene, and *thyP3* gene, which was introduced into strain SB591 by transformation with pFT33, were partially purified by agarose gel electrophoresis. To accomplish this, *B. subtilis* DNA from strain SB1207 (*thyA*$^-$ *thyB*$^+$), SB1141 (*thyA thyB*$^+$), SB1200 (*thyA*$^+$ *thyB*), and SB1163 (*thyA thyB thyP3*$^+$) was digested with *Eco*RI restriction endonuclease. DNA samples (2 to 3 μg) were electrophoresed in agarose gel. The gels were sliced into 2-mm slices, the DNA was extracted from each slice, and the extracted DNA was assayed for its Thy$^+$ transforming activity as described by Harris-Warrick et al. (4). Only two populations of fragment sizes were found to be associated with Thy$^+$ transforming activity: fractions 8 and 9, containing large DNA fragments (>10 Mdal), and fractions 21 and 22, containing DNA fragments of 4.2 Mdal. In DNA from the *thyA*$^+$ *thyB*$^+$ strain, both sets of fractions were biologically active. In *thyA thyB*$^+$ DNA, only the 4.2-Mdal fragments could transform to Thy$^+$. In *thyA*$^+$ *thyB* or *thyA thyB thyP3*$^+$ DNA, only high-molecular-weight fragments had the Thy$^+$ transforming activity. The results indicate that the *thyB* gene is located on a smaller *Eco*RI fragment, whereas both *thyA* and *thyP3* are on high-molecular-weight fragments which are identical in size (within the limits of resolution of the technique used). This

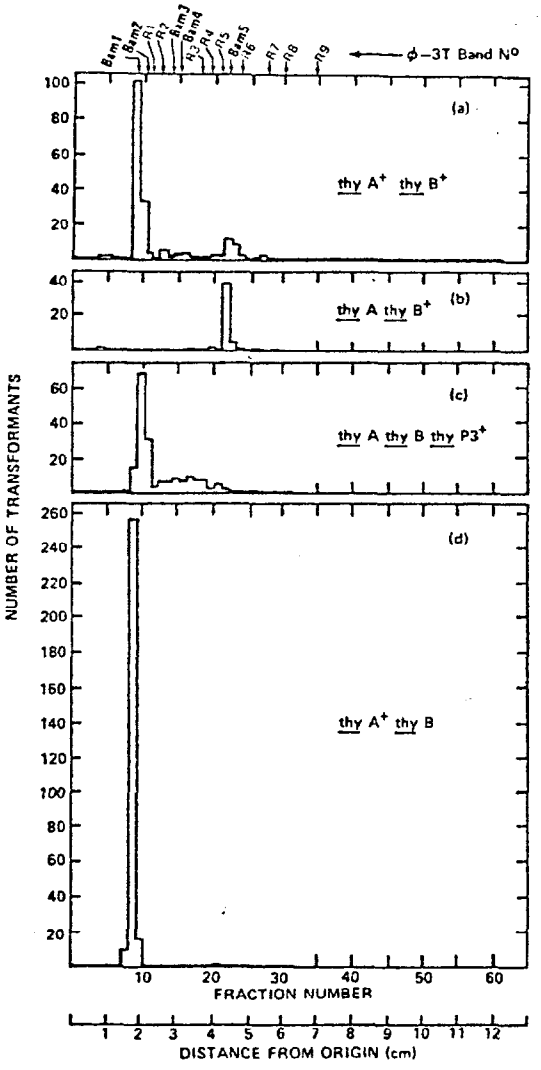

FIG. 2. *Partial purification of restriction fragments containing the bacterial thyA and thyB genes and the ϕ3T thyP3 gene integrated into the bacterial chromosome. The experimental procedure was as described elsewhere (5). After electrophoresis, 0.7% agarose gels containing EcoRI-cleaved DNA from (a) SB1207, (b) SB1141, (c) SB1163, and (d) SB1200 was cut into 2-mm slices. The DNA was extracted and used to transform SB748 (Thy⁻) competent cells. The peaks of Thy⁺ transforming activity are shown as a function of gel slice number. The number of transformants shown is the number of Thy⁺ clones scored per 10^7 cells plated. The positions of the ϕ3T EcoRI and BamHI bands (molecular weight standards) are indicated on the top of the figure.*

was independently verified by testing the phenotype of the Thy⁺ transformants. As expected, all transformants obtained with DNA from fractions 21 and 22 behaved like *thyA thyB*⁺ mutants; that is, they were temperature sensitive and partially resistant to trimethoprim in the presence of thymine. The transformants obtained with DNA from fractions 8 and 9 were like *thyA*⁺ *thyB* mutants; they were resistant to high temperature and sensitive to trimethoprim. These experiments demonstrate that in the case of nine independently constructed *thyP3*⁺ transformants, the phage thymidylate synthetase gene integrated at the *thyA* locus.

The hybridization pattern in channel D of Fig. 1 indicates that in this case (SB1155, constructed by transformation of SB591 with *Bam*HI-cleaved ϕ3T DNA), *thyP3* integrated at a site different from *thyA*. In this channel, a 1.5-Mdal *Hin*dIII band from the SB591 deletion is still visible; in addition, a new 2.1-Mdal band is stained. In this case, the integration of the *thyP3* gene occurred at a site on the SPβ prophage (12) which is known to lysogenize almost all *B. subtilis* 168 strains (Fig. 3). *Eco*RI-cleaved SB1207 DNA (channel A) hybridized cRNA pFT23 to one band only, the *thyA* region. The SPβ lysogen, SB591, digested with *Eco*RI (channel B)

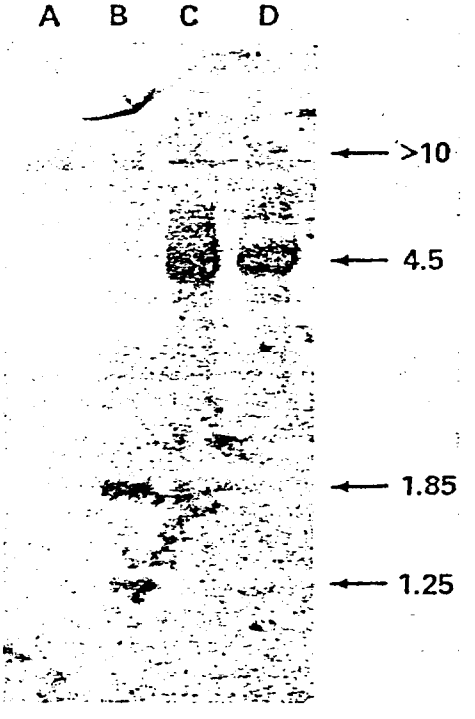

FIG. 3. *BamHI-cleaved ϕ3T DNA recombines with SPβ prophage. EcoRI-cleaved DNA from (A) SB1207, (B) SB591, (C) SB1155, and (D) ϕ3T lysogen of SB1207 was transferred by Southern blotting and hybridized with cRNA pFT23. Sizes of the stained bands are expressed in megadaltons.*

hybridized to this probe at two additional bands (1.85 and 1.25 Mdal). In a strain transformed to thymine independence by *Bam*HI-cleaved φ3T DNA (SB1155 [channel C]), the two *Eco*RI SPβ-specific bands could not be detected, and instead a new fragment was found. This fragment had a size of 4.5 Mdal and was the same as the one observed in an *Eco*RI-cleaved φ3T lysogen of SB1207 (channel D).

To confirm that the integration of the *thyP3* occurred at a site on SPβ prophage, it was shown that SPβ phage induced from strain SB1155 converted thymine auxotrophs of *B. subtilis* to thymine independence upon lysogenization. The test was done by allowing SPβ phage that were released spontaneously into the medium during the growth of SB1155 to infect a lawn of Thy⁻ Spβ⁻ bacteria (SB1219). All SB1219 lysogens selected from the middle of the phage plaques (50 out of 50) were Thy⁺ and had the immunity region of SPβ.

Frequency of the *thyP3* integration at each of the two loci. To measure the relative frequency at which *thyP3* DNA integrates at the *thyA* and SPβ regions, SB591 was transformed with φ3T DNA cleaved by *Eco*RI, *Bgl*II, and *Bam*HI endonucleases. The size of the φ3T DNA segments containing *thyP3* genes was estimated to be 4.5 Mdal for *Eco*RI (2), 4.2 Mdal for *Bgl*II, and 36.6 Mdal for *Bam*HI. Twenty Thy⁺ transformants from each experiment were tested. None of the clones transformed by *Eco*RI or *Bgl*II φ3T DNA released SPβ phage capable of converting the SB1219 auxotroph to thymine prototrophy. In contrast, all (20 of 20) of the *Bam*HI digested φ3T DNA-transformed clones were lysogenic for a recombinant SPβ/φ3T Thy⁺ transducing phage. This suggests that the *thyP3* gene integrates preferentially into the SPβ region when the transforming DNA is *Bam*HI-cleaved φ3T DNA and into other loci (presumably *thyA*) when the transforming DNA is *Eco*RI- or *Bgl*II-digested φ3T DNA.

This suggests that the probability of integration of the *thyP3* gene at each of the two loci depends on the extent of homology shared by the transforming DNA and the recipient region of the chromosome. It can be predicted from this assumption that the transformation efficiency of uncleaved high-molecular-weight φ3T DNA would be greatly reduced if the recipient chromosome were cured of SPβ. That this indeed was the case is shown in Table 2. φ3T DNA transformed an SPβ⁺ strain (SB1219) with an efficiency approximately 20-fold lower than that of an SPβ⁺ strain (SB591). A strain that was doubly lysogenic for φ3T and SPβ and which thus contained twice the homology of SB591 was transformed by φ3T DNA at twice the efficiency of a single lysogen. The transformation efficiency of the small *Eco*RI and *Bgl*II φ3T fragments was the same in both strains, indicating that the probability of crossing over between these segments and their homologous counterparts in SPβ is low.

TABLE 2. *Transformation efficiency of small and large restriction fragments of φ3T in B. subtilis strains*

Recipient Thy⁻ strain	*thy* genes	Transformation efficiency (%)*		
		φ3T DNA (79.1 Mdal)	*Eco*RT φ3T DNA (4.5 Mdal)	*Bgl*II φ3T DNA (4.2 Mdal)
SB1203 (*thyA*, SPβ, φ3T)	*thyA thyB thyP3*	200	NT	NT
SB591 (*thyA*, SPβ)	*thyA thyB*	100	0.5	0.6
SB1219 (*thyA*)	*thyA thyB*	5	0.5	0.5

* Defined as the number of Thy⁺ transformants obtained with φ3T DNA divided by the number of Thy⁺ transformants obtained with SB168 DNA. Transformation efficiency of uncleaved φ3T DNA in the SB591 recipient was arbitrarily designated as 100%, and the results were standardized accordingly. The transformation assays were performed at limiting DNA concentrations, and the numbers shown are the average of five experiments. The competence level (defined as the number of Thy⁺ transformants obtained with SB168 DNA divided by the number of viable transformants) was 0.2% (SB1219), 0.1% (SB591), and 0.005% (SB1203). SB1203 carries a thymineless derivative of φ3T in the SB591 background. The regions homologous to the transforming DNA in the recipient strain are indicated in parentheses.

Comparison of the *thyA* and *thyP3* genes. The two thymidylate synthetase genes are related at the molecular level. This was shown by the hybridization technique and by the ability of *thyP3* DNA to recombine with *thyA* sequences. Since neither of these methods is quantitative, they do not exclude the possibility that *thyP3* is also related to the bacterial *thyB* gene. Therefore, the phenotypic expression of all three genes was tested as an alternative measure of their relatedness. The two thymidylate synthetase genes of *B. subtilis* can be phenotypically distinguished. The phenotype of the *thyP3* gene was tested by growing φ3T lysogens and *B. subtilis* strains transformed with *thyP3* on aa plates (see Materials and Methods) containing trimethoprim (5 μg/ml) and thymine (50 μg/ml) or on aa plates at 37 and 48°C (Table 3). It was found that *thyP3*⁺ strains consistently resembled *thyA*⁺ strains. The phenotypic expression of *thyP3* was not affected by its chromosomal location or φ3T lysogeny.

When hybridization experiments were performed on *Hae*II-digested *B. subtilis* DNA, us-

TABLE 3. *Phenotypic expression of the thyA, thyB, and thyP genes in B. subtilis*[a]

Strain	*thy* genes	Phenotype	1 aa + TRM (5) + Thy (50)	2 aa + TRM (10) + Thy (50)	3 aa + TRM (5)	4 aa + TRM (10)	5 aa, 48°C	6 L, 48°C	7 aa, 37°C	8 L, 37°C
SB1223	*thyA*$^+$ *thyB*	Thy$^+$	−	−	−	−	+	+	+	+
SB1141	*thyA thyB*$^+$	Thy$^+$	+	−	−	−	−	+	+	+
SB168	*thyA*$^+$ *thyB*$^+$	Thy$^+$	−	−	−	−	+	+	+	+
SB591	*thyA thyB*	Thy$^-$	+	+	−	−	−	+	−	+
SB1149	*thyA thyB thyP3*$^+$	Thy$^+$	−	−	−	−	+	+	+	+
SB1123	*thyA*$^+$ *thyB*$^+$ *thyP3*$^+$	Thy$^+$	−	−	−	−	+	+	+	+
SB1151	*thyA thyB thyP3*$^+$	Thy$^+$	−	−	−	−	+	+	+	+
SB1152	*thyA thyB thyP3*$^+$	Thy$^+$	−	−	−	−	+	+	+	+
SB1162	*thyA thyB thyP3*$^+$	Thy$^+$	−	−	−	−	+	+	+	+
SB1165	*thyA thyB thyP3*$^+$	Thy$^+$	−	−	−	−	+	+	+	+
SB1166	*thyA thyB thyP3*$^+$	Thy$^+$	−	−	−	−	+	+	+	+
SB1167	*thyA thyB thyP3*$^+$	Thy$^+$	−	−	−	−	+	+	+	+
SB1168	*thyA thyB thyP3*$^+$	Thy$^+$	−	−	−	−	+	+	+	+
SB1155	*thyA thyB thyP3*$^+$	Thy$^+$	−	−	−	−	+	+	+	+
SB1203	*thyA thyB thyP3*	Thy$^-$	+	+	−	−	−	+	−	+
SB1204	*thyA thyB thyP3*	Thy$^-$	+	+	−	−	−	+	−	+

[a] The growth of bacterial strains in trimethoprim (TRM) was scored in the presence and absence of thymine (Thy) added to aa plates (Spizizen salts [9] supplemented with glucose, agar, and 20 μg each of the common amino acids/ml). TRM and Thy concentrations are indicated in the parentheses (in micrograms per milliliter). The resistance to high temperature was monitored at 48°C on aa plates and as a control on L plates at permissive and nonpermissive temperatures. Strains SB1149, SB1151, SB1152, SB1162, SB1165, SB1167, SB1168, and SB1155 were constructed by lysogenization of SB591 with a φ3T DNA or by transformation of SB591 with DNA from different pFT plasmids. SB1203 and SB1204 were constructed by lysogenization of SB591 with different thymineless mutants of φ3T. SB1123 is a φ3T lysogen in the SB168 background.

ing cRNA pFT *thyP3* as a probe, a DNA sequence difference between *thyP3* and *thyA* was found (Fig. 4).

In wild-type DNA (SB168, SD1207), *thyA* was located on an *Hae*II fragment of 3.7 Mdal. In strain SB591, the deletion had apparently removed one of the *Hae*II sites and fused the *thyA* region to another segment. The *thyA* gene was now found on a larger band with a size of 7.9 Mdal. In this case, integration of the *thyP3* gene at the *thyA* locus (SB1162) did not restore the hybridization pattern of the wild-type DNA. It is argued, therefore, that *thyP3* gene does not contain the *Hae*II recognition sequence, whereas the *thyA* region does. This result suggests that some nucleotide change at the molecular level has occurred between the two genes during evolution.

DISCUSSION

Recent results from this laboratory establish that φ3T shares extensive homology with the *B. subtilis* chromosome (10). At least three different regions of the bacterial genome were shown to be capable of hybridizing RNA complementary to φ3T. These regions include: (i) the *thyA* region which was found to be homologous to the *thyP3* region in φ3T; (ii) SPβ, which is a cryptic temperate bacteriophage of *B. subtilis* strain 168 and which was demonstrated to be a close relative of φ3T (although the SPβ genome does not carry the *thy* gene, it does contain sequences surrounding *thyP3* in φ3T); and (iii) other regions of the *B. subtilis* chromosome. The nature and location of these sequences in the bacterial or phage chromosome were not identified. It was shown, however, that they are not homologous to the *thyP3* gene or the DNA surrounding the *thyP3* gene in φ3T.

This investigation demonstrates that the mechanism of transformation of *B. subtilis* Thy$^-$ strains to thymine prototrophy by φ3T DNA involves site-specific recombination of φ3T sequences with their homologous counterparts in the bacterial chromosome. As a result of this recombination, the *thyP3* gene of φ3T is integrated into the bacterial DNA. Two genetically distinct integration sites were identified: the bacterial *thyA* locus and a site on the SPβ prophage. The attachment site for SPβ prophage lies between *ilvA* and *kauA* (16). The integration of the *thyP3* DNA at the *thyA* locus presumably involves crossing over of the two structural thymidylate synthetase genes. Since sequences homologous to the *thyP3* gene were not detected in SPβ, the insertion of the *thyP3* into SPβ prophage occurs, most likely, by recombination of the sequences surrounding *thyP3* gene with

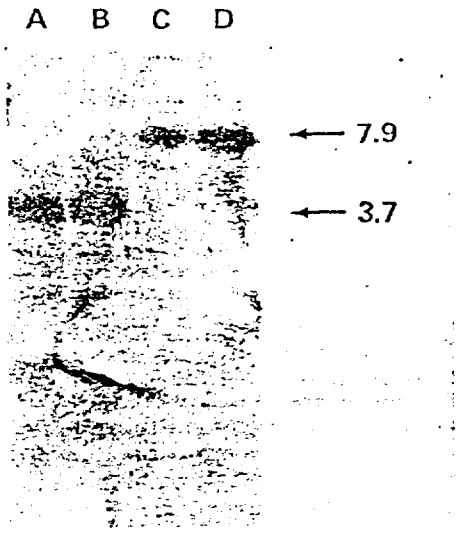

FIG. 4. *DNA sequence differences between the thyA and thyP3 genes. HaeII-cleaved DNA from (A) SB168, (B) SB1207, (C) SB591, and (D) SB1162 was transferred to nitrocellulose filters and hybridized with cRNA pFT thyP3. Sizes of the stained bands are expressed in megadaltons.*

homologous sequences present in SPβ. When *thyP3* integrates at the SPβ site, a recombinant SPβ/ϕ3T phage is created which was shown to carry the immunity region of SPβ.

The frequency of the integration of the *thyP3* gene at each of the two loci and some of the parameters affecting this frequency were studied. It was found that when large ϕ3T fragments (36.6 Mdal; generated by *Bam*HI cleavage of ϕ3T DNA) or intact ϕ3T molecules (79.1 Mdal) were used to transform *B. subtilis* strains, the integration occurred 95% of the time at the site on SPβ prophage. In 5% of the cases, *thyP3* integrated at the *thyA* locus. When the primary integration site was deleted by curing strains of SPβ, the efficiency of transformation dropped, as expected, to 5%. It is presumed that in these transformants the integration occurred at the secondary *thyA* site. When small ϕ3T fragments were used (*Eco*RI, 4.5 Mdal; *Bgl*II, 4.2 Mdal), *thyP3* did not integrate at the SPβ site (none of 20 cases). Instead, the *thyP3* was shown to have recombined with the *thyA* gene (eight of eight cases). As expected, no reduction in transformation efficiency strains cured for SPβ was observed for *Eco*RI and *Bgl*II ϕ3T fragments. These findings are consistent with the assumption that the probability of recombination at each of the sites is positively correlated with the amount of homology shared by the transforming fragment of DNA and the recipient region. This conclusion was further corroborated by the fact that the transformation efficiency by large fragments of ϕ3T DNA in strains doubly lysogenic for SPβ and Thy$^-$ derivatives of ϕ3T is twice that observed for a single lysogen.

No conclusive statement can be made about the history of the *thyP3* gene. However, evidence presented here and in the accompanying paper (10) suggests that SPβ phage acquired a bacterial *thyA* gene by transformation or by recombination with homologous DNA sequences and became an ancestor of ϕ3T and another related phage, ρ11 (1). It can be proposed that ϕ3T (or ρ11) transduced the *thyA* gene into *B. subtilis* from another source. The function of the *thyP* gene in ϕ3T and ρ11 is not clear. It is expressed constitutively in the lysogen, unlike many other genes associated with phage growth.

Regardless of the evolutionary origin of the *thyP3* gene, it is certain that the *thyP3* gene in ϕ3T and the *thyA* gene of *B. subtilis* share a common ancestry. This conclusion is based on the studies of their structure (cross-hybridization and ability to recombine) and phenotypic expression (resistance to trimethoprim in the presence of thymine and to high temperature). At some time, however, molecular evolution has occurred between the two *thy* genes. This was revealed by the minor differences in nucleotide sequences observed during the studies of their restriction enzyme digests.

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LITERATURE CITED

1. **Dean, D. H., J. C. Orrego, K. W. Hutchison, and H. O. Halvorson.** 1976. New temperate phage for *Bacillus subtilis, ρ11*. J. Virol. 20:509–519.
2. **Ehrlich, S. D., H. Bursztyn-Pettegrew, I. Stroynowski, and J. Lederberg.** 1976. Expression of the thymidylate synthetase gene of the *Bacillus subtilis* bacteriophage ϕ3T in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. 73:4145–4149.
3. **Graham, R. S., F. E. Young, and G. A. Wilson.** 1977. Effect of site specific endonuclease digestion on the *thyP3* gene of bacteriophage ϕ3T and the *thyP11* gene of bacteriophage ρ11. Gene 1:169–180.
4. **Harris-Warrick, R. M., Y. Elkana, S. D. Ehrlich, and J. Lederberg.** 1975. Electrophoretic separation of *B. subtilis* genes. Proc. Natl. Acad. Sci. U.S.A. 72:2207–2211.
5. **Harris-Warrick, R. M., and J. Lederberg.** 1978. Inter-

species transformation in *Bacillus*: sequence heterology as the major barrier. J. Bacteriol. 133:1237–1245.
6. Nehard, J., A. R. Price, L. Schack, and E. Thomassen. 1978. Two thymidylate synthetases in *B. subtilis*. Proc. Natl. Acad. Sci. U.S.A. 75:1194–1198.
7. Sinsheimer, R. L. 1977. Recombinant DNA. Annu. Rev. Biochem. 46:415–438.
8. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503–517.
9. Spizizen, J. 1958. Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. Proc. Natl. Acad. Sci. U.S.A. 44:1072–1078.
10. Stroynowski, I. 1981. Distribution of bacteriophage ϕ3T homologous deoxyribonucleic acid sequences in *Bacillus subtilis* 168, related bacteriophages, and other *Bacillus* species. J. Bacteriol. 148:91–100.
11. Tucker, R. G. 1969. Acquisition of thymidylate synthetase activity by a thymine-requiring mutant of *Bacillus subtilis* following infection by the temperate phage ϕ3. J. Gen. Virol. 4:489–504.
12. Warner, F. D., G. A. Kitos, M. P. Romano, and H. E. Hemphill. 1976. Characterization of SPβ: a temperate bacteriophage from *Bacillus subtilis* 168 M. Can. J. Microbiol. 23:45–51.
13. Wilson, M. C., J. C. Farmer, and F. Rothman. 1966. Thymidylate synthesis and aminopterin resistance in *Bacillus subtilis*. J. Bacteriol. 92:186–196.
14. Wilson, G. A., and F. E. Young. 1972. Intergenotic transformation of the *Bacillus subtilis* genospecies. J. Bacteriol. 111:705–716.
15. Young, F. E., M. T. Williams, and G. A. Wilson. 1976. Development of biochemical genetics in *Bacillus subtilis*, p. 5–13. *In* D. Schlessinger (ed.), Microbiology—1976. American Society for Microbiology, Washington, D.C.
16. Zahler, S. A., R. Z. Korman, R. Rosenthal, and H. E. Hemphill. 1977. *Bacillus subtilis* bacteriophage SPβ. Localization of the prophage attachment site and specialized transduction. J. Bacteriol. 129:556–558.